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(54) Title: METHOD FOR THE COVALENT ATTACHMENT OF POLYSACCHARIDES TO PROTEIN MOLECULES

(57) Abstract

Disclosed and claimed are a method for the covalent attachment of poly– and oligosaccharides to protein molecules via hydrogen peroxide depolymerization of the polysaccharide units, followed by attachment of the depolymerized polysaccharide chain to the amino acid groups of a protein of interest through a linker molecule, and products therefrom, and methods for using the products.

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TITLE OF THE INVENTION

METHOD FOR THE COVALENT ATTACHMENT OF POLYSACCHARIDES TO PROTEIN MOLECULES

FIELD OF THE INVENTION

The present invention relates to a method for the covalent attachment of poly- and oligosaccharides to protein molecules via hydrogen peroxide depolymerization of the polysaccharide units, followed by attachment of the depolymerized polysaccharide chain to the amino acid groups of a protein of interest through a linker molecule.

Several publications are referenced in this application. Full citation to these publications is found where cited or at the end of the specification, immediately preceding the claims; and each of these publications is hereby incorporated by reference. These publications relate to the state of the art to which the invention pertains; however, there is no admission that any of these publications is indeed prior art.

BACKGROUND OF THE INVENTION

In recent years, there has been considerable interest in developing approaches to covalently attach poly and oligosaccharides to protein molecules. This approach has been applied in the area of vaccine development, where purified bacterial capsular polysaccharides have been covalently attached to protein molecules (Dick, W. E. et al., 1989). These constructs have been termed conjugate vaccines.

The reason for preparing these constructs is that purified bacterial capsular polysaccharides, which are classified as t-cell independent antigens, can be converted into t-cell-like antigens by covalent attachment to certain protein molecules. Unconjugated polysaccharide vaccines are not capable of eliciting an anamnestic response in man, and the immune response to these antigens can be of limited duration, especially in younger populations. For this reason, the polysaccharide vaccines have not been recommended for usage in infant populations, because of their inherent limited efficacy in this population.

Over the last ten to fifteen years, purified capsular polysaccharide from *Haemophilus influenzae* type b has been covalently attached to a number of protein molecules, e.g. diphtheria toxoid and tetanus toxoid protein, and these conjugates are known to elicit a t-cell dependent immune response in the infant population. This feature has allowed the development and licensure of effective vaccines against disease caused by the bacterium *Haemophilus influenzae* type b (Santosham, M., 1993). This approach of preparing conjugate vaccines has also been extended to other capsular polysaccharides, such as those purified from *Neisseriae meningitidis* and *Streptococcus pneumoniae*.

OBJECTS AND SUMMARY OF THE INVENTION

One general route that has been used to prepare these saccharide-protein conjugates is to activate one or more sites on

the saccharide chain so that these activated sites will react with one or more of the protein's amino acid groups.

In developing a strategy to covalently attach polysaccharides to proteins, the present invention provides a route wherein the polysaccharide chain is initially depolymerized down to oligosaccharides of mean molecular weight in the range of 10-30,000 e.g., 10-25,000 daltons. Two advantages for using depolymerized polysaccharides to prepare the conjugates are: (a) the conjugates prepared from using depolymerized polysaccharides may be inherently more immunogenic than the corresponding conjugates prepared from full length polysaccharides; and (b) reactions used to prepare these conjugate vaccines can offer a higher degree of control, as well as more versatility in process design, when using depolymerized polysaccharide chains versus full length polysaccharide chains.

In some cases, one can covalently attach the depolymerized polysaccharide chains by adding a specific reagent that allows bond formation between the polysaccharide and protein molecules. Depending upon the chemistry that is utilized to perform this operation, one or more bonds can form between the polysaccharide and protein. In other cases, an alternative route has been employed whereby a small chemical molecule is attached to either the depolymerized polysaccharide or protein molecule, and this molecule, because of its inherent reactivity, serves as a linker molecule between the polysaccharide and protein. These

molecules have been termed chemical linkers, linker and/or direct linker.

The method of the present invention preferably utilizes the latter approach, whereby a linker molecule is attached to the polysaccharide chain that affords selective attachment to protein amino acid groups. In this process, polysaccharides are first depolymerized using hydrogen peroxide under mild hydrolytic conditions. The hydrolysis reaction is a well controlled process that yields a uniform distribution of oligosaccharide chains that readily react with a hydrazide and/or an amine. The degree to which the hydrazide or amine can be attached to the hydrogen peroxide hydrolyzed polysaccharides can be increased by addition of a water soluble carbodiimide reagent compound.

The reason for this characteristic is that a certain population of the depolymerized polysaccharide chains possess a chemical group that can be readily derivatized with hydrazide or amine by the addition of water soluble carbodiimides to the reaction medium. These resulting hydrazide/amine derivatized polysaccharide chains can then be selectively attached to protein carboxylic acid groups.

Hence, the method of the present invention provides a process whereby polysaccharides can be controllably degraded or depolymerized under mild hydrolytic conditions, i.e., using low concentrations of hydrogen peroxide at slightly elevated temperatures and at slightly acidic, basic or neutral conditions,

e.g., temperatures in the range of 30-80°C and pH values in the range of 4.5-8.0 ± 0.10.

This process was surprisingly adapted from degradation of carbohydrate molecules by alkaline hydrogen peroxide under an assortment of reaction conditions (Isbell, H.S. et al., 1987). This depolymerization process appears to proceed by a random attack at glycosidic linkages by hydrogen peroxide, thereby yielding a uniform molecular weight distribution of depolymerized carbohydrate chains.

Historically, polysaccharides have been depolymerized by a variety of approaches that include heating under either acidic, basic or neutral conditions, ultrasonic irradiation, shear force, enzyme catalyzed cleavages, radical mediated, metal-ion catalyzed, and periodate oxidation where applicable (Yalpani, M., 1988). The ability of any one of these methods to depolymerize a particular polysaccharide chain is dictated by the physical make-up of the polysaccharide chain. Prediction of the best hydrolytic conditions is, at times, difficult even when one knows the structure of the polysaccharide repeat unit.

However, in unexpected contrast to the historical approaches to depolymerizing polysaccharides, the method of the present invention has been applied to a number of structurally dissimilar polysaccharides.

Defining conditions to obtain the desired molecular weight distribution is a relatively straightforward process,

because the single most influential experimental parameter in the inventive process is temperature. The other experimental parameters that allow for fine adjustments of molecular weight distribution are the percent of hydrogen peroxide used in the reaction mixture and the length of time of the reaction.

A number of mechanisms have been proposed for the alkaline degradation of carbohydrates using hydrogen peroxide (Isbell, H.S. et al., 1987). Cleavage of the chains appears to occur selectively at the glycosidic bond. The reducing end sugar so generated either remains in its native oxidation state (i.e. aldehyde) or may undergo oxidation to the next higher oxidative state (i.e. carboxylic acid). The aldehyde form is much more reactive towards hydrazides than are normal reducing end sugar groups generated by acid or base hydrolysis, which suggests that the reducing end sugar may exist in an open form and not as a hemiacetal.

According to the mechanism proposed by Isbell (1987), the reducing end sugar may undergo limited degradation in these reactions to yield a smaller alditol unit, thereby leaving the reducing end sugar in the open form. The available data supports the assertion that the chains generated by hydrogen peroxide depolymerization are much more reactive towards hydrazides than are chains that are depolymerized by either acid or base.

There are also depolymerized polysaccharide chains that contain groups that are reactive with water soluble

carbodiimides, that allow for further derivatization with either amine or hydrazide containing compounds. One can derivatize both polysaccharide groups in the same reaction by adding the water soluble carbodiimide compound to the reaction medium.

Accordingly, an object of the invention can include any of providing: a method for preparing a construct, the construct comprising a poly and/or oligosaccharide covalently attached to a protein molecule, wherein the method comprises depolymerizing the poly/oligosaccharide using hydrogen peroxide under mild hydrolytic conditions, derivating the depolymerized polysaccharide and/or oligosaccharide with an amine and/or a hydrazide, preferably in the presence of a carbodiimide, and conjugating the derivatized, depolymerized oligo/polysaccharide with a protein molecule; a construct from such a method; a composition such as a therapeutic, immunological or vaccine composition comprising such a construct and optionally a pharmaceutically or verterinarily acceptable carrier or diluent; a method for making such a composition comprising the aforementioned method for preparing the construct and optionally admixing the construct with the carrier or diluent; and, a method for treating an animal (e.g., mammal) or human (including infant) in need of treatment or for inducing an immunological or protective immune response in such an animal or human comprising administering the construct or composition comprising the construct.

Therefore, the present invention provides a method for preparing a construct, the construct comprising a poly and/or oligosaccharide covalently attached to a protein molecule, wherein the method comprises depolymerizing the poly/oligosaccharide using hydrogen peroxide under mild hydrolytic conditions, derivating the depolymerized polysaccharide and/or oligosaccharide with an amine and/or a hydrazide, and conjugating the derivatized, depolymerized oligo/polysaccharide with a protein molecule.

The invention further provides a construct derived from derivatized, depolymerized bacterial capsular polysaccharide selected from the group consisting of *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F and *Neisseria meningitidis* groups A, C, W135 and Y.

The present invention also provides a method wherein the mean molecular weight of the depolymerized poly/oligosaccharide is 10-30,00 daltons, e.g., 10-25,000 daltons.

The invention additionally provides a means for directly linking the derivatized, depolymerized polysaccharide and/or oligosaccharide to a carboxylic acid group of a protein molecule using a water soluble carbodiimide reagent, which reaction may optionally be carried out in the presence of N-hydroxysuccinimide.

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